ORIGINAL ARTICLE

In Vitro Antifungal Susceptibility of Environmental Isolates of *Cryptococcus* spp. from the West Region of Cameroon

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ABSTRACT

BACKGROUND: Cryptococcus neoformans is responsible of cryptococcosis, a life-threatening infection that affects healthy and immunocompromised individuals. It is the first cause of adult acute meningitis in some sub-Saharan African countries with a mortality rate of about 100% in cases of inappropriate therapy. This study aimed at examining the occurrence and the antifungal patterns of Cryptococcus isolates from pigeon droppings and bat guanos in the west region of Cameroon.
METHODS: A total of 350 samples were randomly collected from three selected localities of west region of Cameroon. The identification was performed based on capsule production assessed by Indian ink preparation. Additional tests performed were urea broth, glycine and tryptophan assimilation tests. The antifungal susceptibility test was performed by the broth microdilution method.
RESULTS: Mycological analysis led to the identification of 98 isolates, of which 57 isolates of C. neoformans var. gattii and 41 isolates of C. neoformans var. neoformans. All the isolates showed resistance to antifungals tested except nystatin which showed MIC mean values ranging between 0.5 µg/mL and 0.65 µg/mL.
CONCLUSION: The prevalence of C. neoformans in pigeons and bats excreta in the west region of Cameroon is 28.57 %. C. neoformans var. gattii and C. neoformans var. neoformans are the main serotypes. Isolates found to be resistant to fluconazole and ketoconazole. Our results emphasize the need for further study on the molecular epidemiology in comparison with clinical isolates.
KEYWORDS: Cryptococcus neoformans, bird excreta, antifungal susceptibility

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INTRODUCTION

Cryptococcus neoformans is an encapsulated, ubiquitous environmental yeast that causes cryptococcosis, a potentially serious disease that affects healthy and immunocompromised individuals, especially patients with AIDS (1). It is actually the first cause of adult acute meningitis in some sub-Saharan African countries with a mortality rate of about 100% in cases of inappropriate therapy, and about 20% to 30% in cases of antifungal treatment (1,2). The aetiological agents of cryptococcosis comprise four serotypes grouped into two major species, namely, *Cryptococcus gattii* (serotypes B and C) and *Cryptococcus neoformans*, (serotype A and D) (3). These two species differ in their biochemical characteristics especially their amino acids assimilation ability and clinical characteristics (4,5). *C. neoformans* var. *grubii* is mostly incriminated in infections with immunocompromised hosts while *C. neoformans* var. *gattii* predominantly affects

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immunocompetent hosts (6,7,8). They also differ in their ecological niches and geographical distribution. Globally, the distribution of *Cryptococcus* species in nature is very large and is especially associated to decaying wood of certain species of trees, fruits and bird droppings, particularly pigeon droppings (9). *C. neoformans* is commonly isolated from bird excreta while *C. gattii* has been isolated mainly from samples of eucalyptus and other trees around the world (10). As infection by *C. neoformans* occurs through inhalation of propagules from the environment, bird excreta as a source of infectious propagules could play a central role in the transmission of *C. neoformans* from the environment to humans. Therefore, bird excreta appear as a dangerous reservoir and potential source of inhaled *C. neoformans*. In the west region of Cameroon, pigeons are domesticated and therefore have a very close proximity to humans. In addition, there is an increasing presence of bats in urban areas, colonizing many tree species. Considering the incidence of human cryptococcosis in Cameroon and the fact that birds are becoming more close to humans in some regions, this study was undertaken to determine the prevalence of *C. neoformans* in pigeon droppings and bat guanos in three localities of the west region of Cameroon. Furthermore, the antifungal susceptibility of the isolates was evaluated against four commonly used antifungals.

MATERIALS AND METHODS

Study area and sample collection: The West Region is 13,892 km² of territory located in the central-western portion of the Republic of Cameroon. Its population is estimated to be 1,834,800. Based on the high presence of bats and pigeons, three localities including Bafoussam, Dschang and Santchou were selected for the study.

Isolation and identification of *Cryptococcus* species: Samples were randomly collected. Dry samples were collected with spatulas and placed in identified clean plastic bags. Wet samples were collected with a sterile cotton-tipped swab moistened in sterile saline solution (0.85 % NaCl) supplemented with chloramphenicol (10 µg/ml) and placed in a test tube with 3 ml of the same solution. The collected samples were transported in sterilized plastic bags. The samples were processed following the method described by Soogarun (11), but with slight modifications on the isolation procedure. One gram of each sample was dissolved in 9 mL of sterile physiological saline (0.9% aqueous NaCl) containing 0.4 mg/mL of chloramphenicol, vortexed vigorously for 1-3 min and filtered through sterile gauze. After 10 minutes, 50µL of supernatant was streaked onto Sabouraud's dextrose agar plate containing 0.05 mg/mL of chloramphenicol. Plates were then incubated at 37 °c for 3-21 days. Creamed round brown yeast-like colonies of suspected *C. neoformans* detected were subcultured on new Sabouraud dextrose agar tubes to obtain single colonies.

Colony morphology and microscopic observation of the presence of capsule in India ink preparation were performed. Urease enzyme production was assessed on prepared indole-urea agar culture medium following the manufacturer's protocol. For the biochemical tests, the auxanogram technique was used to differentiate, identify and confirm the specie variety. In fact, on glycine and tryptophan assimilation testing composed media based on the fact that only *C. neoformans var. gattii* isolates are able to use glycine and tryptophan as sole sources of nitrogen (4,5). For the test, 175µL of each composed media (media composition: glucose 20g/L, glycine 20g/L or tryptophan 4g/L and Chloramphenicol 0.05g/L) was introduced in 96 well microtiter plates and 25µL of each solution prepared in concentrations corresponding to 0.5 on the Mac-Farland scale, was added. The plates were then incubated at 37 °c for 48 to 72 hours, and the growth was recorded. *C. neoformans* KN99α (serotype A) was used as reference strain for control.

Antifungal susceptibility testing: In addition to the reference clinical strains *C. neoformans* KN99α, ten isolates of each variety were randomly selected and tested for their antifungal susceptibility to four commonly used antifungals: namely, nystatin, amphotericin B, fluconazole and ketoconazole. The minimum inhibitory concentration was determined as recommended by the NCCLS (12). MIC values were interpreted as described by Lozano-Chiu *et al*. and Nguyen and Yu respectively (13,14) (Table 1).

Table 1: Minimum inhibitory concentration **(**MIC) values interpretation

Antifungals	MIC range (µg/mL)		
	Sensible	Intermediately susceptible	Resistant
Fluconazole	≤ 8	16- 32	≥ 64
AmphotericinB	≤ 1	2- 4	> 4
Ketoconazole	≤ 0,125	0.25- 0.5	≥ 1
Nystatin	≤ 1	2- 4	> 4

RESULTS

A total of 350 samples were collected over a period of eight months, from April to November 2015. They were made up of 200 samples of pigeon droppings and 150 samples of bat guanos (Figure 1). From the 350 samples collected, 103 yeast-like *Cryptococcus* were isolated among which 101 showed capsules in the India ink preparation. The 101 India ink positive isolates subjected to urease enzyme test revealed 100 positive and 01 negative. Therefore, 100 isolates were confirmed as *Cryptococcus neoformans* isolates (Table 2). Glycine and tryptophan assimilation results showed that, among the 100 isolates positive to urease, 57 were of the *Cryptococcus gattii* variety, while 41 were of the *Cryptococcus neoformans* var *neoformans* (*Cryptococcus neoformans*) variety and 02 of the isolates remained undetermined (Table 3). The antifungal results are presented in (Table 4). Compared to the reference clinical strain (MICs ranged from 4 µg/mL to 32 µg/mL), the antifungal susceptibility results showed high resistance of isolates to azole antifungals. The MICs values ranged from 16 µg/mL to > 256 µg/mL and from 8 µg/mL to 64 µg/mL for fluconazole and ketoconazole respectively.

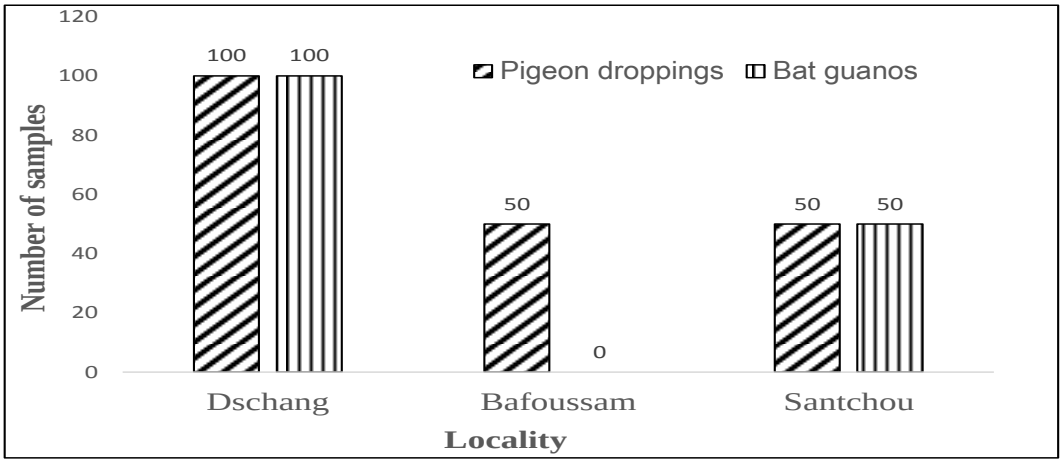

Figure 1*: Distribution of samples according to the collection areas*

Table 2: Results of culture and microscopic observation in India ink

	Types of excreta	**Culture (n=350)**	**%**	**India ink (n=103)**	**%**	**Urease test (n=101)**	**%**	**Total**	**%**
	PD	56	16	56	54.37	55	54.45	**55**	55
Positive	BG	47	13.42	45	43.68	45	44.55	**45**	45
	Total	**103**	**29.42**	**101**	**98,05**	**100**	99	**100**	100
	PD	144	41.14	0	0	1	1		
Negative	BG	103	29.44	2	1.95	0	0		
	Total	247	**70.58**	**2**	**1.95**	**1**	1		

PD= Pigeon dropping; BG= Bat guanos

Table 3: Glycine and tryptophan assimilation results and interpretation

	Positive		Total	Negative		Total
Amino acid	**PD (n=55)**	**BG (n=45)**		**PD (n=55)**	**BG (n=45)**	
Gly	24	18	42	31	27	58
Trp	23	19	42	32	26	58
Trp+Gly	23	18	**41**	31	26	**57**
Interpretation						
Cn var *neoformans*				31	26	**57**
Cn var g*attii*	23	18	**41**			
Undetermined	1	1	**2**			

Cn= Cryptococcus neoformans; PD= Pigeon dropping; BG=Bat guano; Gly=glycine; Trp=tryptophane

Table 4: Antifungal susceptibility of isolates and a reference clinical strain determined by the minimum inhibitory concentration (MIC).

		MIC(µg/mL)		
		Environmental isolates		**Reference clinical strain**
Antifungals		***Cn* var *neoformans* (n=10)**	***Cn* var *gattii* (n=10)**	***Cn KN99α***
Fluconazole	Range	64 - >256	16 - >256	2
	Mean	-	-	
Ketoconazole	Range	16 - 64	8 - 64	1
	Mean	36.8	27.2	
Amphotericin B	Range	4 - 8	64 - 128	2
	Mean	4.6	89.6	
Nystatin	Range	0.125-0.5	0.5 - 1	0.5
	Mean	0.35	0.65	

Cn= Cryptococcus neoformans, n= number of isolates

DISCUSSION

The incidence of opportunistic fungal infections has increased in recent years and is considered as an important public health problem. Several studies have shown that *C. neoformans* remains viable in the dried excrement of birds (15). Therefore bird excreta appear as a dangerous reservoir and potential source of *C. neoformans* infection. In this regard, 350 samples of pigeon droppings and bat guanos were collected in the west region of Cameroon and the isolates obtained were examined for their antifungal susceptibility.

In this study, we observed an occurrence of *C. neoformans* in 100 out of the 350 samples analysed, indicating an incidence of 28.57 % of this yeast in the studied region. This result confirms the presence of this yeast in the environment, in the west region of Cameroon. Similar studies carried out in Nigeria and Jordan showed prevalences of 22.0% (39/177) and 33.3% (336/1009) respectively (16,17). However, in the above studies, the authors used only pigeon dropping and materials under canopies of eucalyptus trees for isolation, not bat guanos. In our study, the number of isolates obtained from pigeon droppings (55%) was higher than those recovered from bat guanos (45%), confirming pigeon droppings as an important reservoir of *C. neoformans.* Previous studies have described pigeon droppings as the main source of *C. neoformans* isolation (18). Nevertheless, with 45% of *C. neoformans* recovered from bat guanos, bats may also play an important role in the

epidemiology of cryptococcosis through their migratory characters. The presence of the two main varieties of *C. neoformans*, namely *C. neoformans var. neoformans* and *C. neoformans var. gattii* in the west region of Cameroon confirm the geographical distribution of *C. neoformans* as described in several previous studies (15, 5, 4, 19). In fact, most of these previous studies showed that *C. neoformans var. neoformans* has a worldwide distribution while *C. neoformans var. gattii* is associated with decaying wood in tropical and sub-tropical regions of the world. Nevertheless, Overy *et al* (20) identified the presence of *Cryptococcus gattii* in Canada showing that this pathogen colonizes other parts of the world.

All the isolates tested showed resistance to fluconazole, ketoconazole and amphotericin B antifungals. Such a pattern of susceptibility from environmental *C. neoformans* isolates was not found in the literature. Most studies reported that environmental isolates of *C. neoformans* were susceptible to these antifungals (21,22). Gutch *et al* (23), using the microdilution method, verified the susceptibility profile of clinical and environmental isolates of *Cryptococcus neoformans* and *Cryptococcus gattii* in Jabalpur, a city of Madhya Pradesh in Central India. Although low sensitivity of environmental isolates to fluconazole has been demonstrated by Rossi *et al* (24), these results may draw the attention of clinician researchers on a prospective emergence of resistant clinical isolates since contamination can easily occur by inhalation of propagules from the environment. Contrary to amphotericin B, our isolates appeared to be susceptible to nystatin which is also an antifungal belonging to the polyene class.

This study revealed that the prevalence of *C. neoformans* in the excreta of pigeons and bats in the west region of Cameroon is 28.57%, *C. neoformans* var. *gattii* and *C. neoformans* var. *neoformans* being the two main representative serotypes. Isolates were found to be resistant to fluconazole and ketoconazole. Considering the incidence of human cryptococcosis in Cameroon, especially in HIV patients, and the fact that pigeons and bats are widely spread birds in these localities, our results emphasize the need for further studies on the molecular epidemiology in comparison with clinical isolates.

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REFERENCES

1. Dromer F, Lortholary O. Cryptococcose. *EMC –Maladies Infectieuses*, 2003; 8-613-A-10, 10.
2. Gullo FP, Rossi SA, Sardi Jde C, Teodoro VL, Mendes-Giannini MJ, Fusco-Almeida AM. Cryptococcosis: epidemiology, fungal resistance, and new alternatives for treatment. *Eur J Clin Microbiol Infect Dis*. 2013; 32(11):1377-91.
3. Kwon-Chung KJ, Fraser JA, Doering TL, Wang Z, Janbon G, Idnurm A, Bahn YS. *Cryptococcus neoformans* and *Cryptococcus gattii*, the etiologic agents of cryptococcosis. *Cold Spring Harb Perspect Med*. 2014; 4(7): a019760.
4. Chan MY, Tay ST. Enzymatic characterisation of clinical isolates of *Cryptococcus neoformans*, *Cryptococcus gattii* and other environmental *Cryptococcus* spp. *Mycoses*. 2010; 53(1):26-31
5. Chaskes S, Frases S, Cammer M, Gerfen G, Casadevall A. Growth and pigment production on D-Tryptophan medium by *Cryptococcus gattii*, *Cryptococcus neoformans*, and Candida albicans . *J Clin Microbiol*. 2008; 46: 255-264.
6. Mseddi F, Sellami A, Jarboui MA, Sellami H, Makni F, Ayadi A. First environmental isolations of *Cryptococcus neoformans* and *Cryptococcus gattii* in Tunisia and review of published studies on environmental isolations in Africa. *Mycopathologia*. 2011; 171(5):355-60.
7. Chowdhary A, Rhandhawa HS, Prakash A, Meis JF. Environmental prevalence of *Cryptococcus neoformans* and *Cryptococcus gattii* in India: an update. *Crit Rev Microbiol*. 2012; 38(1):1-16.
8. Idnurm A, Lin X. Rising to the challenge of multiple *Cryptococcus* species and the

diseases they cause. *Fungal Genet Biol*. 2015; 78:1-6.

9. Mseddi F, Sellami A, Jarboui MA, Sellami H, Makni F, Ayadi A. First environmental isolations of *Cryptococcus neoformans* and *Cryptococcus gattii* in Tunisia and review of published studies on environmental isolations in Africa. *Mycopathologia*. 2011; 171(5):355-60
10. Leite DP Jr, Amadio JV, Martins ER et al. *Cryptococcus* spp isolated from dust microhabitat in Brazilian libraries. *J Occup Med Toxicol*. 2012; 7(1):11.
11. Soogarun S, Wiwanitkit V, Palasuwan A et al. Detection of *Cryptococcus neoformans* in bird excreta. Southeast *Asian J Trop Med Public Health*. 2006; 37(4):768-70.
12. National committee for Clinical Laboratory Standard (N.C.C.L.S). Reference method for broth dilution Antifungal susuptibility Testing of yeasts. Approved Standard M27-A2. Wayne, USA. 17, 9, 2002.
13. Therese K L, Bagyalakshmi R, Madhavan HN, Deepa P. (2006). In-vitro susceptibility testing by agar dilution method to determine the minimum inhibitory concentrations of amphotericin B, fluconazole and ketoconazole against ocular fungal isolates. *Indian J Med Microbiol*. 2006; 24(4):273-9.
14. EUCAST. European Committee on Antimicrobial Susceptibility Testing Antifungal Agents Breakpoint tables for interpretation of MICs Version 6.1. 2013.
15. Casadevall A, Perfect, JR. Ecology of *Cryptococcus neoformans*. Washington, Asm Press, 1998.
16. Nweze E, Kechia F, Dibua U, Eze C, and Onoja U. Isolation of *Cryptococcus neoformans* from environmental samples collected in Southeastern Nigeria. *Rev. Inst. Med. Trop. Sao Paulo*. 2015; 57(4): 295-8.
17. Akram M, Sinasi T, Ahmet G, Mehmet A, and Levent. *Cryptococcus neoformans* varieties from material under the canopies of eucalyptus trees and pigeon dropping samples from four major cities in Jordan. *Mycopathologia*. 2004; 158: 195-199.
18. Li AS, Pan WH, Wu SX, Hideaki T, Guo NR, Shen YN, Lu GX, Pan RG, Zhu MC, Chen M, Shi WM, Liao WQ. Ecological surveys of the *Cryptococcus* species complex in China. *Chin Med J (Engl)*. 2012; 125(3):511-6
19. Overy DP, McBurney S, Muckle A, Lund L, Lewis PJ, Strang R. *Cryptococcus gattii* VGIIb-like Variant in White-Tailed Deer, Nova Scotia, Canada. *Emerg Infect Dis*. 2016; 22(6):1131-3
20. Aade-Silva L, Ferreira-Paim K, Mora DJ, Da Silva PR, Andrade AA, Araujo NE, Pedrosa AL, Silva-Vergara ML. Susceptibility profile of clinical and environmental isolates of *Cryptococcus neoformans* and *Cryptococcus gattii* in Uberaba, Minas Gerais, Brazil. *Med Mycol*. 2013; 51(6): 635-640
21. Pedroso RS, Lavrador MAS, Ferreira JC, Candido RC, Maffei CML. *Cryptococcus neoformans* var. *grubii* Pathogenicity of environmental isolates correlated to virulence factors, susceptibility to fluconazole and molecular profile. *Mem Inst Oswaldo Cruz*. 2010; 105(8): 993-1000
22. Andrade-Silva L, Ferreira-Paim K, Mora DJ, Da Silva PR, Andrade AA, Araujo NE, Pedrosa AL, Silva-Vergara ML. Susceptibility profile of clinical and environmental isolates of *Cryptococcus neoformans* and *Cryptococcus gattii* in Uberaba, Minas Gerais, *Brazil. Med Mycol*. 2013; 51(6):635-40.
23. Gutch RS, Nawange SR, Singh SM, Yadu R, Tiwari A, Gumasta R, Kavishwar A. Antifungal susceptibility of clinical and environmental *Cryptococcus neoformans* and *Cryptococcus gattii* isolates in Jabalpur, a city of Madhya Pradesh in Central India. *Braz J Microbiol*. 2015; 46(4):1125-33.
24. Rossi SA, Trevijano-Contador N, Scorzoni L, Mesa-Arango AC, de Oliveira HC, Werther K, de Freitas Raso T, Mendes-Giannini MJ, Zaragoza O, Fusco-Almeida AM. Impact of Resistance to Fluconazole on Virulence and Morphological Aspects of *Cryptococcus neoformans* and *Cryptococcus gattii* Isolates. Front Microbiol. 2016; 7:153.